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Critical Role of Water in the Binding of Volatile Anesthetics to Proteins

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Abstract

Numerous small molecules exhibit drug-like properties by low-affinity binding to proteins. Such binding is known to be influenced by water, the detailed picture of which, however, remains unclear. One particular example is the controversial role of water in the binding of general anesthetics to proteins as an essential step in general anesthesia. Here we demonstrate that a critical amount of hydration water is a prerequisite for anesthetic-protein binding. Using nuclear magnetic resonance, the concurrent adsorption of hydration water and bound anesthetics on model proteins are simultaneously measured. Halothane binding on proteins can only take place after protein hydration reaches a threshold hydration level of ~0.31 gram of water per gram of proteins at the relative water vapor pressure of ~0.95. Similar dependence on hydration is also observed for several other proteins. The ratio of anesthetic partial pressures at which two different anesthetics reach the same fractional load is correlated with the anesthetic potency. The binding of nonimmobilizers, which are structurally similar to known anesthetics but unable to produce anesthesia, does not occur even after the proteins are fully hydrated. Our results provide the first unambiguous experimental evidence that water is absolutely required to enable anesthetic-protein interactions, shedding new light on the general mechanism of molecular recognition and binding.

Keywords

interfacial water; anesthetic-protein interaction; protein hydration

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ASSOCIATED CONTENT

Supporting Information. Further discussions on anesthetic adsorption on proteins and the surface areas of proteins, and Fig. S1-S3 are included in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

1. INTRODUCTION

Water is an active player in processes ranging from the assembly of small apolar molecules[1-2] to protein folding and functioning[3-4]. Water may also mediate the interaction between a ligand and a receptor,[5-8] the understanding of which can potentially accelerate the design of new drugs. The water-mediated interaction is particularly important for small apolar molecules that exhibit drug-like action through low-affinity binding to proteins. A large class of such small molecules, known as general anesthetics,[9-10] can induce reversible biological effects such as unconsciousness, amnesia, analgesia, and immobility.

The role that water plays in the binding of general anesthetics to proteins has been an issue of controversy for over a half century.[11-12] One view believes that the entropy-driven hydrophobic interaction is the dominant force for anesthetic-protein binding.[13] This is based on the facts that both general anesthetics and their sites of action, which are thought to be certain pre-existing or inducible protein cavities in the central nervous system (CNS), are largely hydrophobic.[14-15] A competing viewpoint, based on the dominant enthalpy contribution from the thermodynamical measurement,[16-18] believes that direct anesthetic-protein interactions, such as the van de Waals and weak electrostatic attraction, are responsible for the binding. There are also observations that are incompatible with either of the above binding mechanisms, pointing to the critical role of water instead.[19-21] For instance, several studies have shown that anesthetic binding sites are amphipathic in nature, with possible accessibility to the aqueous phase.[22-24] There also exists a class of molecules, known as nonimmobilizers, that do not cause anesthesia at predicted concentration despite of their structure similarities to known anesthetics. It was noticed that nonimmobilizers have much lower water solubility than their anesthetics counterparts, suggesting that a minimal hydrophilicity is required for being anesthetics.[25-26]

More specifically, water molecules have been found near the binding sites in the high-resolution crystal structure of anesthetic-protein complexes,[27-28] and during the large-scale molecular dynamics simulations with explicit water molecules.[19-21] The close proximity of these water molecules to both protein surface and anesthetics suggests that the role of water in anesthetic-protein binding must be understood in conjunction with how water approaches the protein surface, *i.e.* the protein hydration process.[29-30] In addition, the effect of hydration water is often obscured by its fast exchange with the vast majority of bulk water and by tedious methods for quantifying bound anesthetics in experiments performed under aqueous condition.[31-32] Since the bulk water is only relevant for the water-water interaction, we can remove the bulk water and control protein hydration very precisely by using water vapor.[29-30] On the molecular level, the role that hydration water plays in anesthetic-protein interaction is independent of the phase of the bulk as either liquid or vapor. On the other hand, the adsorption of drug molecules on proteins is a direct indication of drug-protein interactions.[13] The adsorption of both water and anesthetic molecules on proteins from their respective vapor phase could be competitive, cooperative, or independent, which can reveal the role that water plays in the binding of anesthetic to proteins.

The concurrent adsorption of water and relatively small amount of anesthetics has prevented the adsorption measurement by traditional methods, such as gravimetric or volumetric methods, which can only measure one adsorbent at a time. In this work, the binding of volatile anesthetics is measured *in situ* as a function of protein hydration level using the NMR-based isotherm measurement technique.[33-34] The hydration water and adsorbed fluorinated anesthetics can be separately quantified by ^{1}H and ^{19}F NMR spectroscopy, taken as functions of partial pressures of water vapor and anesthetics.[33-34] Bovine serum albumin (BSA), which has known binding pockets for volatile anesthetics,[31, 35] is used as a model protein. We show that even with the pre-existing binding sites, anesthetic-protein binding

can only take place after protein hydration reaches a threshold level and nonimmobilizer-protein binding does not occur even after the protein is fully hydrated. These results demonstrate the critical role of water in anesthetic-protein interaction as well as apolar ligand recognition and binding in general,[36-38] shedding new light on the mechanism of action of general anesthetics.

2. MATERIALS ANS METHODS

BSA (lyophilized powder, >96%), hen egg white lysozyme (HEWL, 3× crystallized, dialyzed, and lyophilized), and halothane (≥99%) were purchased from Sigma Aldrich. 1-chloro-1, 2, 2-trifluorocyclobutane (F3, 97%), 1,2-dichlorohexafluorocyclobutane (F6, 97%), and 2,3-dichlorooctafluorobutane (F8, 97%) were purchased from Alfa Aesar. Isoflurane (99%) and 1, 2-dimethylperfluorocyclobutane (F12, 97%) were purchased from Indofine Chemical. All materials were used without further purification.

Proteins of ~150 mg in lyophilized powder form were loaded into the quartz NMR sample tube connected to an *in situ* water and anesthetic vapor loading system with controlled vapor pressure.[33-34] The proteins were dynamically pumped for more than 12 hours to remove the hydration water contained in the as-received sample. The dry proteins were then exposed to a certain vapor pressure of anesthetics at room temperature for adsorption study. For the adsorption on partially hydrated proteins, proteins were first exposed to water vapor to a certain hydration level before exposed to anesthetic vapor.

A single pulse of ~3 Is was used to excite the ^{1}H NMR signal at 7 T (300 MHz ^{1}H NMR frequency) to determine the amount of water as described elsewhere.[34] The amount of anesthetics was determined by the ^{19}F NMR signal excited by a single pulse of ~4 μs at 285 MHz ^{19}F NMR frequency. Proton decoupling was not applied during the acquisition of ^{19}F NMR spectra. The concurrent adsorption of water and anesthetics on proteins reached equilibrium within ~12 hours as monitored by NMR signal. The absolute quantity of adsorbed anesthetics was calibrated by comparing the integrated area under the broad peaks in ^{19}F NMR spectra with that under the spectra from the anesthetic vapor of known pressure and volume. The effect of detection delay has been taken into account which was discussed in details elsewhere.[34] For the purpose of identifying the signatures of adsorbed anesthetics and calibrating their quantity, proton decoupling is not necessary for its effective removal of line broadening due to the ^{1}H-^{19}F interaction. Such NMR determined quantity of small molecules adsorbed on surfaces have been shown as a reliable way of isotherm measurement.[33-34, 39]

3. RESULTS AND DISCUSSION

Figure 1A shows the ^{19}F NMR spectrum of the anesthetic halothane at its saturated vapor pressure (P/P_0=1) exposed *in situ* to dry BSA powder. It consists of one narrow peak of Lorentzian shape with full-width-at-half-maximum (FWHM) of ~3 ppm and is identical to that of free halothane (Fig. 1A) in an empty NMR tube. The narrow NMR lineshape is a signature of fast molecular dynamics in the vapor phase, suggesting that the dynamics of halothane molecules is not affected significantly by the halothane-dry BSA interactions. This is also the case when interactions of dry BSA with anesthetics isoflurane (Fig. 1B) and F3 (Fig. 1C) were investigated. In contrast, as shown in Fig. 1D, the line shape changes dramatically when BSA was first hydrated to the hydration level of h~0.33 and then exposed to halothane at P/P_0=1. The hydration level, h, is in the unit of grams of water per gram of proteins. Here, the spectrum consists of a narrow peak of ~3 ppm FWHM and a broad peak of ~25 ppm FWHM. The broad component is a signature of anesthetic adsorption as the result of its interaction with partially hydrated proteins.[31, 35] This is because anesthetic

adsorption is associated with anesthetic molecules approaching and residing on protein surface during the residence time and a highly restricted molecular dynamics for adsorbed anesthetics.[40] The NMR shift of adsorbed molecules can be dominated by the local magnetic susceptibility near protein surface, and this shift varies from one location on proteins to another.[39] Each ^{19}F nuclear spin within the same adsorbed molecule may experience different local magnetic field. The NMR spectrum of adsorbed molecules is therefore broadened by the local magnetic susceptibility. In addition, intramolecular interactions, such as ^{19}F-^{19}F dipolar interaction and the chemical shift anisotropy, are no longer averaged out to zero when the molecular dynamics is highly restricted, also leading to much broader NMR spectrum. In either case, the appearance of a broad component in the ^{19}F NMR spectrum serves as an excellent signature of anesthetic adsorption. Isoflurane (Fig. 1E) and F3 (Fig. 1F) also exhibit strong interactions with hydrated BSA at h>0.3. Figure 1G shows the ^{19}F NMR spectrum of the nonimmobilizers F8[25-26, 41] at P/P_0=1 exposed in situ to hydrated BSA at h=0.33 (red line). Comparing this to the very similar ^{19}F NMR spectrum of F8 in an empty NMR tube (black line in Fig. 1G), it is obvious that no sign of adsorption on proteins (line broadening in NMR spectra) is observed even when the BSA is hydrated to a sufficiently high level of h>0.3. Similar behaviors are observed for nonimmobilizers of F12 (Fig. 1H) and F6 (Fig. 1I).[25-26, 41] Clearly, the water-enabled adsorption of anesthetics on proteins does not work for nonimmobilizers.

While the clinically relevant concentrations were used in the search for potential anesthetic targets,[42] understanding the binding mechanism requires the full range of anesthetic vapor pressure. The direct separation of free and adsorbed anesthetics in NMR spectra allows the *in situ* isotherm measurements of anesthetics adsorption on partially hydrated proteins. Figure 2A shows adsorption isotherms of halothane, isoflurane, and F3 on BSA at different hydration levels. There are dramatic differences in isotherms for adsorption at low (h<0.3) and high (h>0.3) hydration levels. While the adsorption of anesthetics was not observed at h<0.3, the isotherms at high hydration levels (h>0.3) agree well with previous adsorption measurements: X-ray study identified 3 and 8 halothane binding sites at the halothane pressure of 4.8 kPa and 19 kPa, respectively;[27] isoflurane adsorption in BSA solution was determined by the gas chromatographic partition analysis.[31, 35] It is also clear that the anesthetic adsorption at h>0.3 is not the consequence of anesthetic solvation in the interfacial water. The amount of hydration water at h~0.33 can at most dissolve 0.4 halothane, 0.3 isoflurane, and 0.2 F3 molecule for each BSA molecule, estimated from the solubility of halothane (18.0 mM), isoflurane (13.8 mM), and F3 (7.5 mM) in bulk water.[25, 43] The low solubility also suggests that the direct water-anesthetic interaction is also too weak to induce anesthetic adsorption directly on the interfacial water. These ruled out the possibility that anesthetics are dissolved in or adsorbed on water patches itself that is has been adsorbed on protein surface. It is the anesthetic-protein interactions that allow a higher anesthetic concentration near the interface, leading to an apparent higher solubility in protein solution than that in water.[44-45] The adsorption of anesthetics was also observed on a smaller globular protein, HEWL (Fig. S1). The ratio of adsorbed anesthetics on each HEWL to that on each BSA is not proportional to the ratio of surface area of each protein. This suggest that the adsorption of anesthetic on proteins is not due to the mechanism of surface adsorption that demonstrates the proportionality between the amount of adsorbed molecules and available surface area (SI Text). The adsorption of anesthetics on proteins is therefore a direct indication of anesthetics-protein binding with the presence of hydration water (SI Text).

Adsorption isotherms of anesthetics on proteins at high hydration levels (h>0.3) also agree with the relative potencies of different anesthetics. From the adsorption isotherms of halothane, isoflurane, and F3 shown in Fig. 2A, the ratios of anesthetic partial pressures ($P_{\text{isoflurane}}/P_{\text{halothane}}$ and $P_{\text{F3}}/P_{\text{halothane}}$) at which the two anesthetics reach the same level of

fractional loading are determined and are displayed in Fig. 2B. The fractional anesthetic adsorption is the number of adsorbed molecules divided by the maximum number of adsorbed molecules measured at $P=P_0$. Over the full range of adsorption, the average pressure ratios are 1.5±0.1 for $P_{isoflurane}/P_{halothane}$ (mean ± standard deviation), and 2.1 ± 0.4 for $P_{F3}/P_{halothane}$, consistent with anesthetic partial pressure needed to anesthetize 50% of subjects, or minimum alveolar concentration ($MAC_{isoflurane}$: $MAC_{halothane}$ = 1.53: 1 and MAC_{F3}: $MAC_{halothane}$ = 1.87: 1),[25, 46] of the respective anesthetic. Such a quantitative agreement, together with the absence of nonimmobilizer adsorption, suggests that the molecular adsorption on partially hydrated proteins can serve as a parameter that correlates better with the anesthetic potency than the bulk lipid solubility. The same correlation is also confirmed in HEWL (Fig. S2). These results indicate that anesthetic adsorption on globular proteins reflects the key binding characters of the anesthetic action.[10]

Anesthetic adsorption on protein at different hydration levels provides direct evidence that water is critically involved in the anesthetic-protein interactions. Figure 3 depicts the amount of the adsorbed anesthetic at the vapor pressure of ~1.3 kPa versus the hydration level of proteins. Based on the hydration-dependent halothane adsorption on BSA, an obvious hydration threshold can be clearly defined at h~0.31, only above which significant adsorption can be observed. The hydration-dependent adsorption of isoflurane and F3 are both consistent with such a threshold. Anesthetic adsorption on HEWL also shows similar dependence on the hydration level (Fig. S3).

This threshold originates from the intrinsic property of the anesthetic binding sites[20] and the protein hydration process.[29-30, 34] The water adsorption isotherms on BSA and HEWL are shown in Fig. 4. It is known that water is primarily adsorbed to charged and hydrophilic groups at low hydration levels (h<0.2).[29-30, 34] Such hydration water is away from the anesthetic binding sites as the latter is largely hydrophobic in nature.[27] Above h=0.2, most of the protein properties, including the conformation and flexibility, become identical to those in dilute solutions.[34] This ensures that the anesthetic binding sites resume the same configuration as identified in solution.[31, 35] The fact that no anesthetic adsorption occurs at h=0.2 suggests that the direct anesthetic-protein interactions, including van der Waals and electrostatic forces, are too weak to dominate anesthetic adsorption. A very high relatively pressure of water vapor (P/P_0~0.95 for BSA and P/P_0~0.9 for HEWL) is required for the proteins to reach the threshold hydration level of h~0.31 for halothane and other anesthetic binding to proteins (Fig. 4). Such a relative pressure suggests that the chemical potential of water directly involved in anesthetic binding is only slightly lower than that of bulk water that condensates from the saturation vapor at P/P_0=1. Certain hydration water that is adsorbed at this high vapor pressure is critically involved in the binding of anesthetics to proteins.

The thermodynamical contribution from water showed in the concurrent adsorption of both water and anesthetics on proteins provides important evidence that water plays a critical role in anesthetic-protein binding. A molecular-level understanding on the specific role of water may require intensive theoretical simulations with explicit water molecules and thermodynamical details. Nevertheless, our experimental results agree with the theoretical prediction on the role of water in the ligand-cavity recognition.[36-38] These theoretical studies showed that only a small free energy change is required for a model hydrophobic pocket to withdraw water from the bulk.[36-38] These water involved in binding would only be adsorbed on proteins at high vapor pressure, say around ~0.9, corresponding to the threshold hydration level of ~0.3 for anesthetic-protein interaction. It is also known that the entropy of mixing of water with proteins compensates for the increase of enthalpy at this hydration level, leading to the hydration of the anesthetic binding sites.[34] This suggests that once the anesthetic binding sites are hydrated, it is thermodynamically favorable for anesthetics to

replace water within the binding sites.[20] The released water upon anesthetic binding could form more hydrogen bonds with the surrounding hydration water and reduce the free energy of the system.[36] The newly formed hydration-bonded network provides a 'water cap' that further stabilizes the anesthetic molecule within the hydrophobic pocket via the interaction of water with the relative more 'hydrophilic' groups of anesthetics.[37] These processes require a critical amount of hydration water in the proximity of the hydrophobic pocket, a condition that can be achieved only at high hydration level such as h~0.31.[29-30, 34] The threshold hydration level may therefore represents the critical amount of water needed to establish favorable free energy landscape for the binding of anesthetics to the pre-existing binding sites. In contrast, the replacement of hydration water by the hydrophobic nonimmobilizers is unfavorable both entropically and enthalpically for the systems of hydrated protein and the nonimmobilizers, due to the weak interaction between water and nonimmobilizers. Further theoretical and experimental evidence are needed to fully understand the role water plays in preventing the binding of nonimmobilizers to proteins.[23, 41, 47]

4. CONCLUSIONS

In summary, we experimentally demonstrated the critical role that water plays in anesthetic-protein binding. A threshold level of protein hydration is observed to be a prerequisite of anesthetic-protein binding. Such a threshold suggests that a critical amount of water must present in the proximity of the largely hydrophobic anesthetic binding sites before the anesthetic-protein binding can take place. It can be inferred from our results that in integral channel proteins, in which the functionally relevant anesthetic binding occurs at either intra-subunit sites[28] or inter-subunit sites[48] within and outside the membrane, a critical amount of water molecules must be able to access to the anesthetic binding sites in order for the anesthetics to produce their anesthetic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

BSA	bovine serum albumin
HEWL	hen egg white lysozyme
F3	1-chloro-1, 2, 2-trifluorocyclobutane
F6	1,2-dichlorohexafluorocyclobutane
F8	2,3-dichlorooctafluorobutane
F12	1, 2-dimethylperfluorocyclobutane

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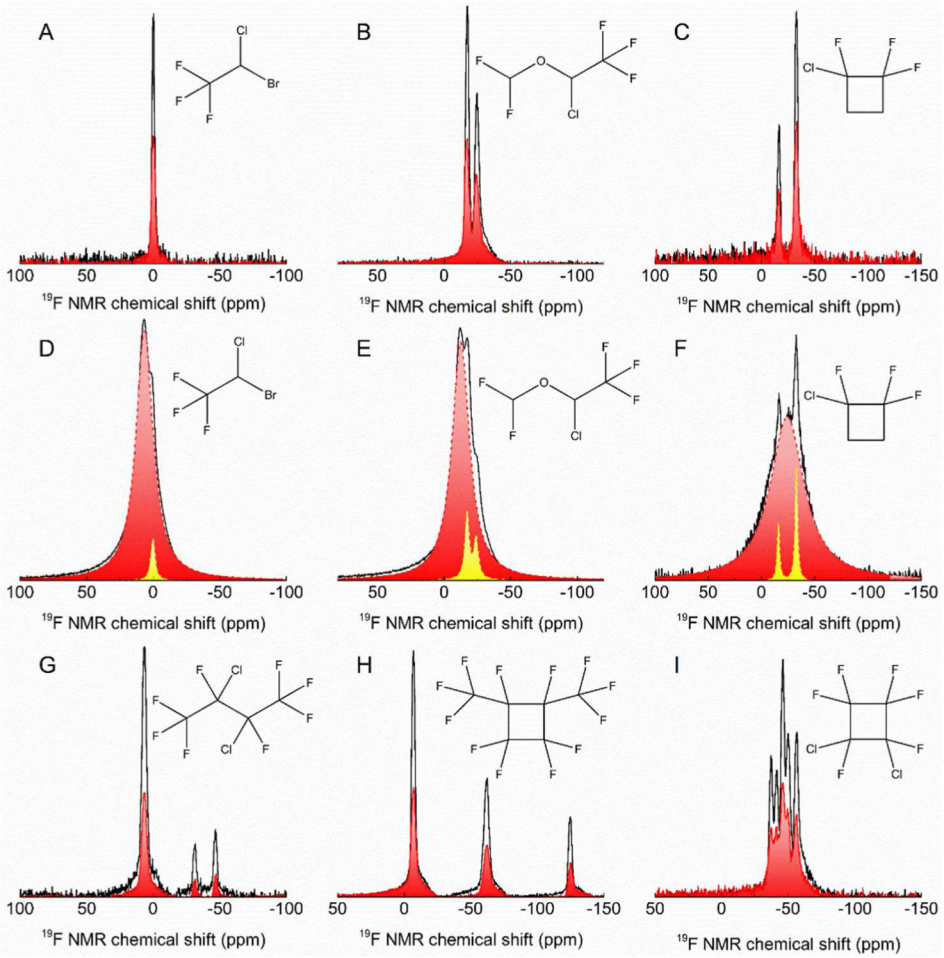

Figure 1.

^{19}F NMR spectra of anesthetics and nonimmobilizers at their respective saturated vapor pressure with dry and partially hydrated BSA (*h*>0.3). **A**-**C**, ^{19}F NMR spectra of anesthetics halothane (**A**), isoflurane (**B**), and F3 (**C**), respectively, exposed to the empty NMR tube (black lines) and to dry BSA (red lines). **D**-**F**, ^{19}F NMR spectra (black lines) of anesthetics exposed to hydrated BSA at *h*>0.3 (**D**: halothane *h*=0.33; **E**: isoflurane, *h*=0.45; and **F**: F3, *h*=0.36). The spectra consist of a broad peak (red dashed lines, Lorentzian fit) in addition to the free anesthetic peak (yellow dashed lines). **G**-**I**, ^{19}F NMR spectra of nonimmobilizers exposed to hydrated BSA at *h*>0.3 (red lines) (**G**: F8, *h*=0.33; **H**: F12, *h*=0.34; **I**: F6, *h*=0.35) in comparison to that exposed to the empty NMR tube (black lines). The peaks from the vapors of anesthetics and nonimmobilizers are used for *in situ* shift reference.

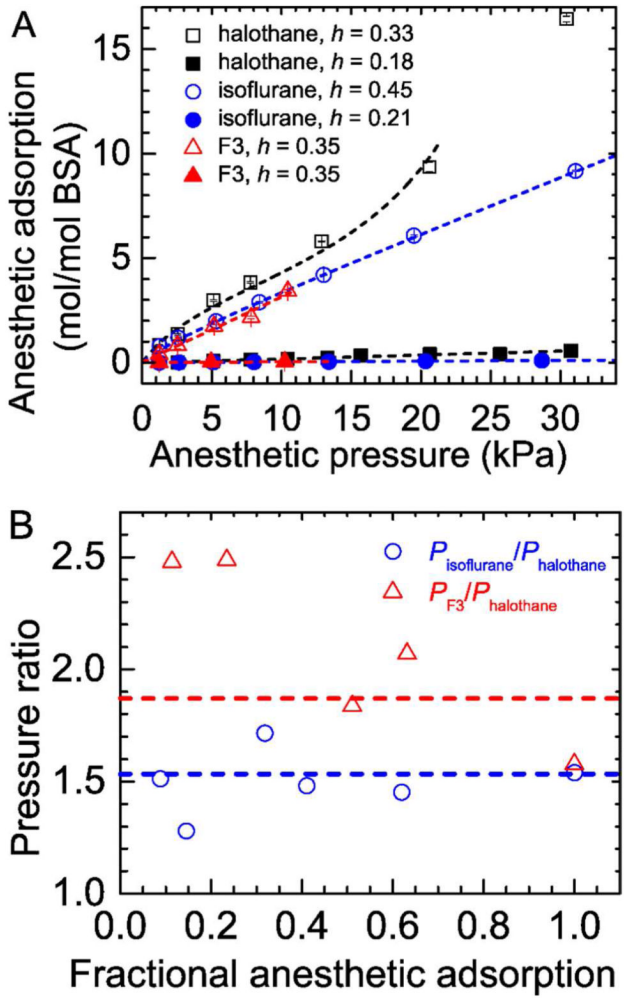

Figure 2.

Anesthetic adsorption isotherms on partially hydrated BSA. **A**, Adsorption isotherms of halothane (open black square: h=0.33, solid black square: h=0.18), isoflurane (open blue circle: h=0.45, solid blue circle: h=0.21), and F3 (open red triangle: h=0.35, solid red triangle: h=0.20), on BSA at different hydration levels. Error bars are the size of the symbols. The dashed lines are guides to eyes. **B**, The pressure ratio at which the same number of different anesthetics, (blue circle: $P_{\text{isoflurane}}/P_{\text{halothane}}$, red triangle: $P_{\text{F3}}/P_{\text{halothane}}$), were adsorbed on proteins at high hydration levels (h>0.3). The pressure ratio is compared to the relative MAC of different anesthetics (blue dashed line $\text{MAC}_{\text{isoflurane}}$: $\text{MAC}_{\text{halothane}}$ = 1.53: 1, red dashed line MAC_{F3}: $\text{MAC}_{\text{halothane}}$ = 1.87: 1). Fractional anesthetic adsorption represents the number of adsorbed isoflurane and F3 divided by the maximum number measured for the respective anesthetic at P=P_0.

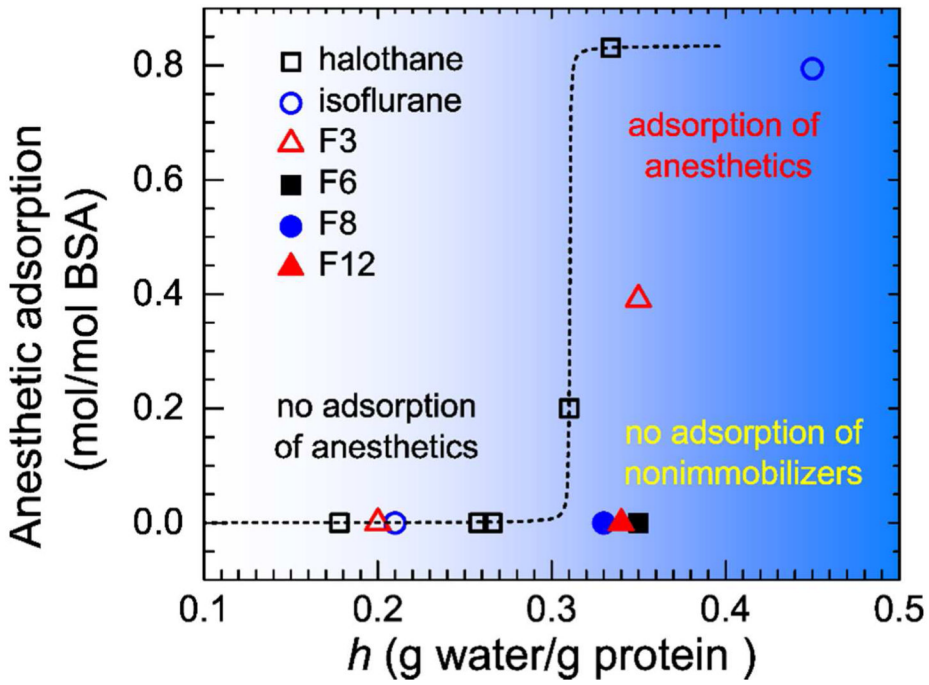

Figure 3.

Effect of water on the anesthetic and nonimmobilizer adsorption on BSA. The amount of adsorbed anesthetics, halothane (open black square), isoflurane (open blue circle), and F3 (open red triangle), at anesthetic vapor pressure of ~1.3 kPa, and nonimmobilizers, F6 (solid black square), F8 (solid blue circle) and F12 (solid red triangle), at their respective saturated vapor pressure. A threshold in hydration level ~0.31 is apparent for anesthetic adsorption on BSA, whereas no such threshold exists that would enable adsorption of nonimmobilizers onto BSA.

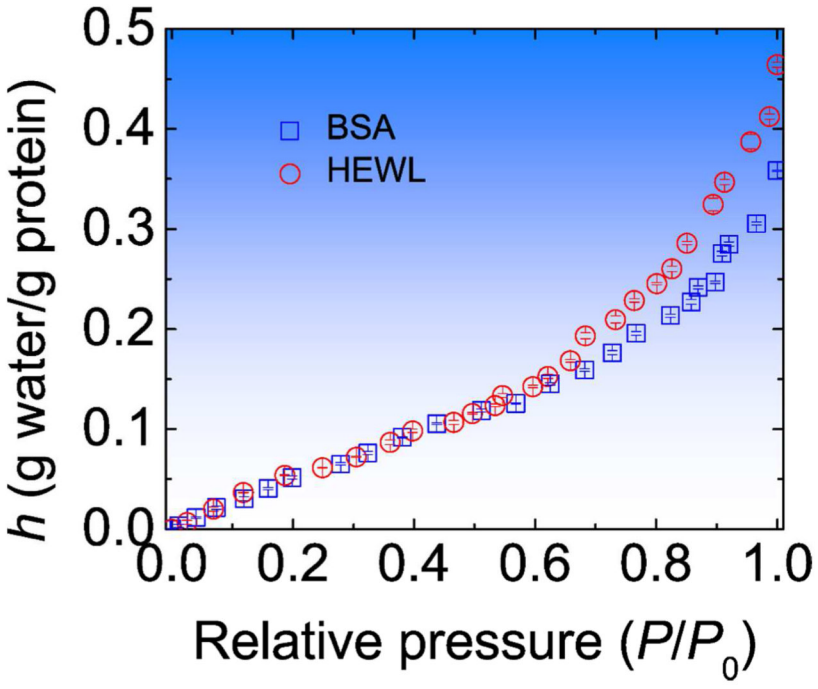

Figure 4.
Water sorption isotherms on BSA (blue square) and HEWL (red circle) at room temperature. The error bars are the standard errors propagated from the conversion of NMR signal intensity to the hydration level as described in details elsewhere.[34]